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# Bone marrow injection stimulates hepatic ductular reactions in the absence of injury via macrophage-mediated TWEAK signaling

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Tissue progenitor cells are an attractive target for regenerative therapy. In various organs, bone marrow cell (BMC) therapy has shown promising preliminary results, but to date no definite mechanism has been demonstrated to account for the observed benefit in organ regeneration. Tissue injury and regeneration is invariably accompanied by macrophage infiltration, but their influence upon the progenitor cells is incompletely understood, and direct signaling pathways may be obscured by the multiple roles of macrophages during organ injury. We therefore examined a model without injury; a single i.v. injection of unfractionated BMCs in healthy mice. This induced ductular reactions (DRs) in healthy mice. We demonstrate that macrophages within the unfractionated BMCs are responsible for the production of DRs, engrafting in the recipient liver and localizing to the DRs. Engrafted macrophages produce the cytokine TWEAK (TNF-like weak inducer of apoptosis) *in situ*. We go on to show that recombinant TWEAK activates DRs and that BMC mediated DRs are TWEAK dependent. DRs are accompanied by liver growth, occur in the absence of liver tissue injury and hepatic progenitor cells can be isolated from the livers of mice with DRs. Overall these results reveal a hitherto undescribed mechanism linking macrophage infiltration to DRs in the liver and highlight a rationale for macrophage derived cell therapy in regenerative medicine.

liver regeneration | adult stem cell

Tissue progenitor cells show promise as a regenerative therapeutic target in a variety of organs (1, 2). Within the liver, hepatocytes regenerate the liver efficiently but following severe injury hepatocyte regeneration may fail and a second regenerative compartment becomes activated. Ductular reactions (DRs) are seen in the liver and contain the population of hepatic progenitor cells (HPCs). DRs have been used to describe the activated biliary epithelial cells and their associated inflammatory reaction. Here we will use the term DR to refer to the epithelial component alone. HPCs are bipotential adult stem-like cells that are thought to reside in the smallest branches of the biliary tree and are defined through their capacity to differentiate under clonogenic conditions *in vitro* into hepatocytes and biliary epithelial cells (3, 4). DRs are found in almost all major forms of human liver disease, as well as in a variety of animal models of both chronic and severe acute liver injury (5). A correlation exists between the extent of liver disease and magnitude of accompanying DRs (6, 7). The therapeutic manipulation of DRs and HPCs offers the potential to regenerate severely injured liver without recourse to liver transplantation (8). Whereas transplantation of HPCs has been successful in rodents (9), isolation of human HPCs is not a practical therapeutic option (10). Manipulation of the liver's endogenous DRs and HPCs represents a more realistic therapeutic approach (11, 12).

There is no accepted marker specific for HPCs as the markers that have been used to isolate HPCs are also expressed upon bile duct cells, and DRs. Cytokeratins (CK) 7 and 19, EpCAM (Epithelial Cell Adhesion Molecule), Dlk1 (Delta Like Homolog),

MIC1-1C3, and Sox9 [sex determining region Y (SRY) box] are all accepted, albeit unspecific, markers of DRs, and have been used to define essential mediators, including many cytokines, which control the DR response to experimental liver injury (5, 13–15). Among the most recently identified of these paracrine signals is TNF-like weak inducer of apoptosis (TWEAK), a cytokine of the TNF family, which directly stimulates proliferation of DRs *in vivo* and HPCs *in vitro* (16, 17).

During liver injury, macrophages are recruited from the bone marrow (BM) to engraft within the liver adjacent to DRs (18). Regeneration of any organ in response to injury is accompanied by macrophage infiltration, which is believed primarily to clear cellular debris. However, following tissue injury macrophages have other roles in tissue repair, which include a role in influencing stem and progenitor cell behavior (4, 19).

Related work in other organs has reported that even in the absence of their own prolonged engraftment, BM-derived cells may stimulate tissue progenitor cells, a process that is speculated to be reliant on paracrine signaling (20). Nonetheless, interpretation of a direct paracrine pathway from BM-derived cells to tissue progenitor cells is complicated during tissue injury by the association of local inflammation and tissue remodeling, processes that are affected by BM-derived cells (21, 22) and that also indirectly influence tissue progenitor cell activation (23).

The BM represents a readily available, autologous source of cells for cell therapy. Recent studies in patients with liver injury have suggested that i.v. delivery of whole bone marrow cells (BMCs) may have beneficial effects, potentially improving liver regeneration and function (2). Earlier reports that BMCs could themselves adopt an epithelial phenotype in the liver have proven to be unfounded (24). A recent study by us in a chronic liver injury model has shown infusion of BM-derived macrophages improves fibrosis and is associated with improved liver function (22). However, a direct effect of macrophages upon the DRs or HPCs themselves has not been shown. The reduction of liver fibrosis itself is associated with improved hepatic regeneration (25) and the degree of matrix remodeling correlates to the magnitude of the DR response (23). Furthermore macrophages themselves are critical for the reduction in fibrosis (21). All these factors mean that the study of a direct effect of macrophages upon DRs becomes near impossible in the setting of chronic liver injury.

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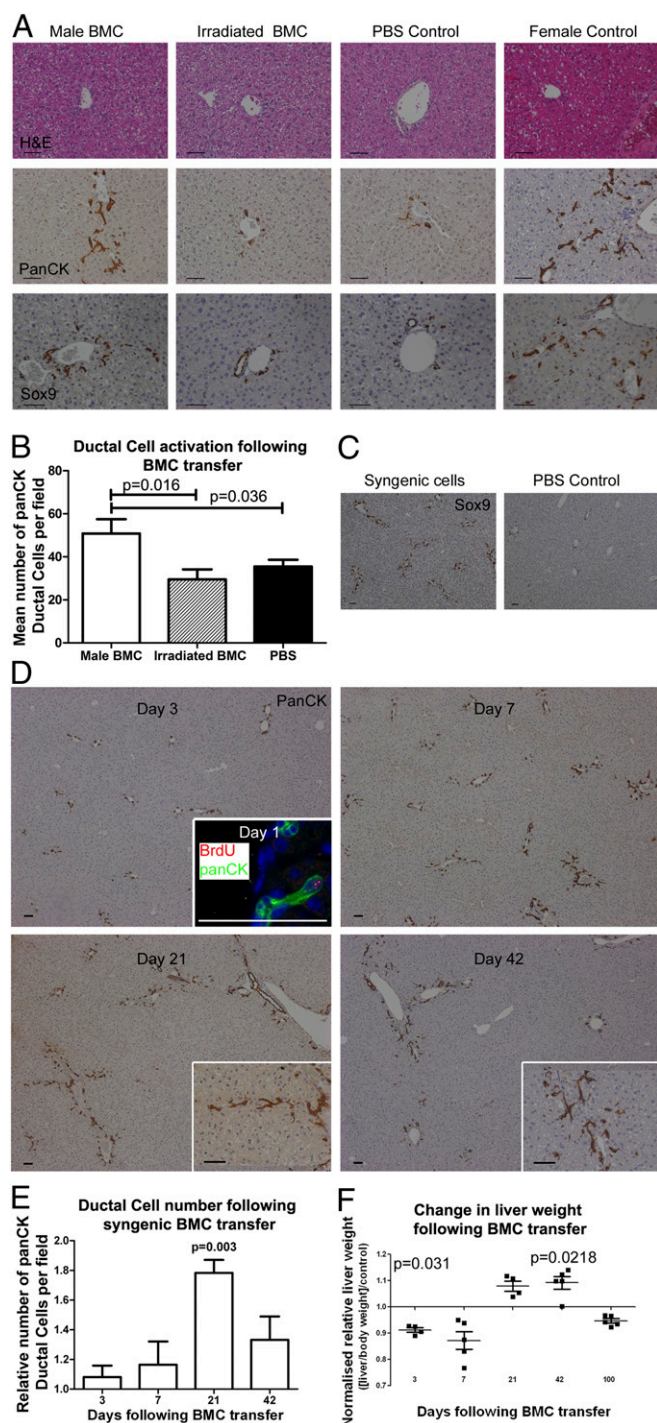
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**Fig. 1.** Ductular reactions occur following bone marrow cell (BMC) transfer. (A) Absence of injury with simultaneous expansion of panCK<sup>+</sup>/Sox9<sup>+</sup> DRs in female mouse liver 42 d following tail vein injection of  $10^7$  BMCs, additional ductular markers (EpcAM and Dlk1 shown in Fig. S1A). Male BMC transfer experiments were repeated four times, each with  $n = 5$  animals per group. (B) Quantification of DRs 42 d after transfer of  $10^7$  male BMCs. (C) Sox9<sup>+</sup> (21 d) and (D) PanCK<sup>+</sup> DRs in inbred female recipients of syngenic BMC transfer with corresponding DR quantification over time (E). Proliferation of DRs is seen as early as 1 d following cell transfer (Inset BrdU 2 h before tissue harvest), with progressive expansion over subsequent 3 wk. Data are presented as relative change in cells per 200 $\times$  magnification field (experimental mean/time matched control mean)  $\pm$  SEM. Mean panCK<sup>+</sup> ductular cells  $34.0 \pm 2.4$ ,  $36.1 \pm 4.9$ ,  $41.9 \pm 2.1$ , and  $35.6 \pm 4.2$  for days 3, 7, 21, and 42, respectively following BMC transfer. (F) Initial reduction in liver size is followed by increase in relative liver weight (liver weight/body weight)/(control

Therefore, we investigated the existence of a paracrine stimulation directly by BMCs upon ductular cells in normal murine liver. Here we report that BMC transfer activates the proliferation of DRs in the absence of liver injury. This has enabled the analysis of the direct effect of macrophages upon DRs in the absence of confounding matrix changes, inflammation, and tissue damage. This DR activation is due to paracrine, macrophage-derived, TWEAK signaling. The understanding of both the signaling mechanisms mediating DR activation, and the cell type responsible for this, may enable the development of novel therapies to stimulate liver regeneration.

## Results

### Transfer of Syngenic BMCs to Healthy Mice Results in Ductular Activation in Healthy Recipient Mice.

To allow tracking of donor-derived BMCs in uninjured recipient liver tissue, initial observations were made in female mice that received a single transfer of  $10^7$  unfractionated BMCs from male donors. These animals showed a consistent, periportal expansion of panCK<sup>+</sup>, Dlk1<sup>+</sup>, EpCAM<sup>+</sup>, and Sox9<sup>+</sup> DRs, from day 7 post transfer, which lasted for up to 6 wk (Fig. 1A and Fig. S1A). No evidence of hepatocellular injury or inflammation was observed in recipient livers at any time point (Fig. 1A and Fig. S1A). Irradiation of BMCs before injection rendered them incapable of inducing ductular activation (Fig. 1A and B and Fig. S1A), implying a requirement for functionally intact BMCs and arguing against the involvement of either a bystander reaction to debris or immunogenic antigens via a minor histocompatibility reaction. Syngenic (inbred female to female) cell transfer yielded an identical ductular activation to that observed in sex-mismatched transfer experiments (Fig. 1C). Again, ductular expansion began as early as 1 d following transfer and progressed over 3–6 wk (Fig. 1D and E).

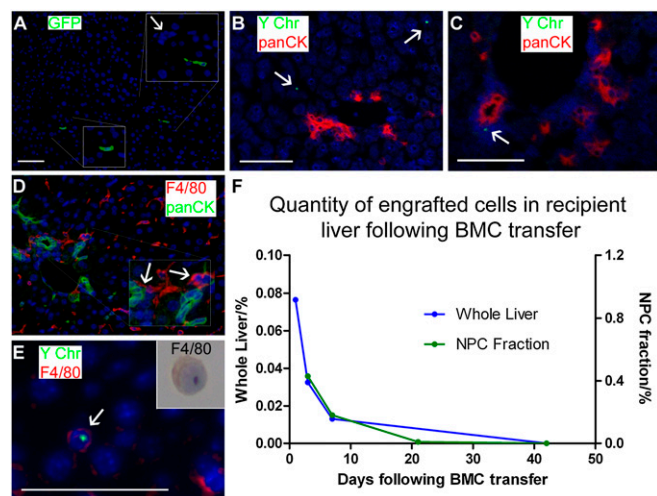
Although liver weight fell initially following BMC transfer, relative liver size increased in association with ductular expansion, resulting in consistently larger livers 6 wk following BMC therapy versus PBS control (Fig. 1F). Whole liver albumin gene expression increased following BMC transfer, although later this gradually normalized (Fig. S1B). No long-term changes were seen in livers of mice followed for 400 d following BMC transfer (Fig. S1C). Identical ductular expansion was observed when BMCs from aged donors was delivered to healthy young mice (Fig. S1D and E).

### BM Transfer Results in Transient Liver Engraftment by Donor-Derived Macrophages.

Two independent cell-tracking strategies were used to study BMC fate in recipient livers: Y chromosome detection in a sex-mismatched BMC therapy and GFP mismatch, whereby GFP<sup>+</sup> donor BMCs were delivered to WT littermates. Both analyses found transient engraftment in the liver by donor-derived cells, with no evidence of transdifferentiation of donor BMCs into panCK<sup>+</sup> DRs or hepatocytes (Fig. 2). Six hours and 1 d following transfer, donor cells were seen throughout all zones of the hepatic lobule with no specific localization (Fig. 2A and B, respectively). From day 3, however, donor-derived cells were found directly adjacent to DRs and make physical contact with the panCK<sup>+</sup> DRs (Fig. 2C), at which time F4/80<sup>+</sup> macrophages also make contact with panCK<sup>+</sup> DRs (Fig. 2D). To characterize the phenotype of engrafted donor-derived cells, FACS selection of GFP<sup>+</sup> cells in WT liver was performed 3 d after BMC delivery from GFP<sup>+</sup> donors. The resultant cell isolate was exclusively CD45<sup>+</sup> (Fig. S2C) and by majority F4/80<sup>+</sup> macrophages (81%); this was confirmed by combining in situ hybridization for the Y chromosome and F4/80 immunostaining (Fig. 2E). Donor cells were consistently undetectable in recipient livers by 42 d post-injection (Fig. 2F). Therefore, the expansion of panCK<sup>+</sup> DRs occurred principally following the loss of engrafted donor-derived cells.

liver weight/body weight) over the period of ductular expansion ( $n \geq 4$  each group). Data are presented as mean number of cells per field  $\pm$  SEM. *P* values denote Student *t* test result vs. control.





**Fig. 2.** Transfer of BMCs leads to transient hepatic engraftment of macrophages. (A) Engraftment of GFP<sup>+</sup> cells within the liver 6 h following tail vein injection of  $10^7$  GFP<sup>+</sup> cells into WT mice. Portal tract highlighted by arrow. (B) One day following sex-mismatched BMC transfer, donor-derived cells remain scattered through hepatic parenchyma. Confocal analysis of recipients using panCK immunohistochemistry (red) and Y chromosome FISH (green, white arrows) with DAPI (blue). (C) Confocal analysis 3 d following sex-mismatched BMC transfer reveal association of donor-derived cells with panCK<sup>+</sup> ductular cells. No evidence of transdifferentiation of donor cells to hepatocytes or ductular cells (A–C). Male and female controls (Fig. S2 A and B). (D) Dual immunohistochemistry for F4/80 (red) and panCK (green) confirms their association 3 d following BMC transfer. (E) Donor-derived cells (Y chromosome, green) express macrophage markers (F4/80, red) on microscopical analysis 3 d following sex-mismatched BMC transfer. Inset: representative F4/80 immunocytochemistry performed on GFP<sup>+</sup> cells sorted by FACS from the nonparenchymal fraction of digested liver 3 d following GFP-mismatched BMC transfer. (F) Quantification of engrafted donor cells from whole liver by real-time PCR of genomic DNA in sex-mismatched BMC transfer model and in the nonparenchymal cell (NPC) fraction by FACS for GFP in GFP-mismatched BMC transfer (Fig. S2C). PCR data (blue) is presented as group mean  $n = 2$  at each time point. FACS data (green) are presented as fraction of the NPC population; each data point represents pooled cell populations,  $n = 5$ .

### Macrophages Are Themselves Capable of Activating a Ductular Reaction.

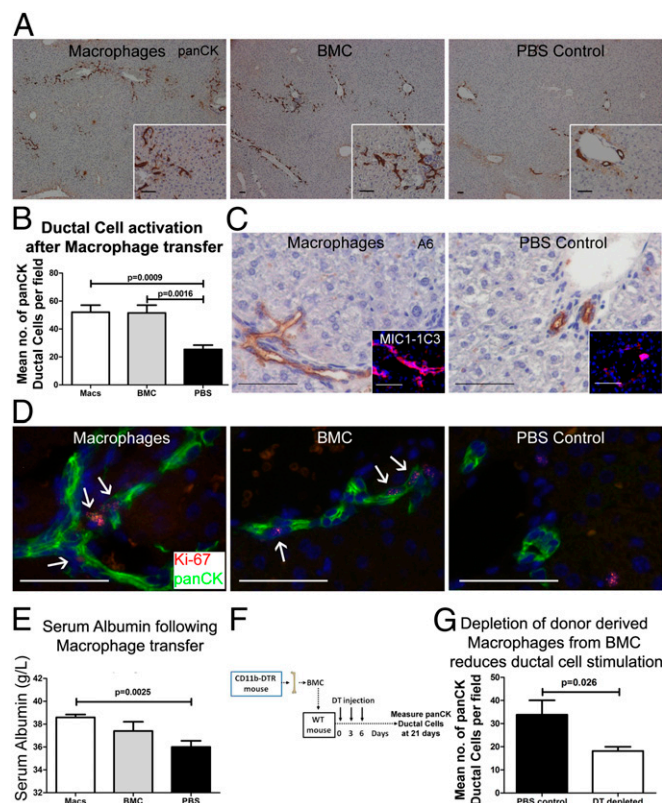
The experiments described above suggest a central role for macrophages in the activation of DRs following BMC injection. We therefore hypothesized that *in vitro* matured macrophages would stimulate a similar DR response to whole BMCs. Macrophage cell transfer resulted in activation of DR proliferation, analogous to that seen with BMCs (Fig. 3 A–D and Fig. S3D), once again in the absence of hepatocellular injury (Fig. S3 E and F). A raised serum alkaline phosphatase together with expression of other biliary/DR marker genes was also observed in mice receiving macrophage transfer (Fig. S3 G–L). Increased serum albumin was observed 21 d following macrophage cell transfer (Fig. 3E).

To examine the role of the macrophage subpopulation within whole BM, whole BMCs were isolated from donor CD11b-diphtheria toxin (DT) receptor (DTR) mice and transferred to healthy WT littermates. DT was then used to selectively deplete donor-derived CD11b<sup>+</sup> macrophages from the transferred population; then the resultant DR response in recipients was measured (Fig. 3F). Sole depletion of donor CD11b<sup>+</sup> macrophages from whole BMCs resulted in reduction of recipient panCK<sup>+</sup> DR expansion (Fig. 3G). At 21 d, a time when donor cells were undetectable, host liver macrophage numbers significantly increased (Fig. S3M).

### Functional Effects of DR Activation Following Macrophage Transfer.

While demonstrating expansion of cells expressing DR markers *in vivo*, we wished to functionally observe the effect of these expanded DRs containing putative HPCs following macrophage/

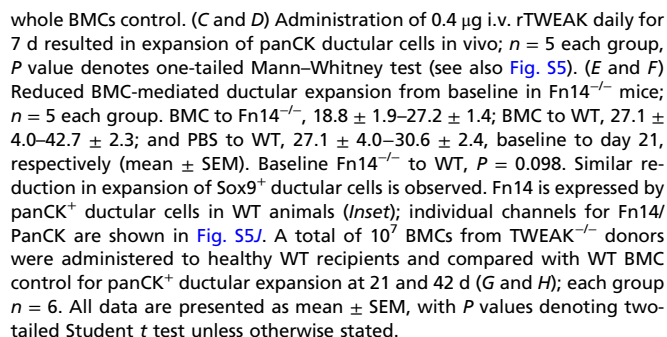
BMC transfer. At the time of previously observed liver enlargement following BMC transfer, Sox9 staining revealed multiple larger cells with hepatocyte-like morphology and Sox9<sup>+</sup> nuclei (Fig. 4A) adjacent to smaller Sox9<sup>+</sup> DR cells, implying differentiation of DRs into functional parenchyma. In agreement with the proliferation and differentiation of DRs into hepatocytes, BrdU label was taken up by DRs 21 d following macrophage transfer (Fig. 4B). A further 4 d later evidence of both retention of BrdU label within clusters of DRs was observed (Fig. 4C) together with BrdU label in both ductular cells and hepatocytes adjacent to one another (Fig. 4D and E). A functional assessment for the presence of HPCs was performed 3 wk following macrophage cell transfer (Fig. 4F). Using FACS, Ter119<sup>−</sup>/CD31<sup>−</sup>/CD45<sup>−</sup>/CD24<sup>+</sup>/EpCAM<sup>+</sup> cells were isolated (Fig. 4F). This population was significantly increased (approximately threefold) in healthy livers following macrophage cell transfer vs. PBS control (total pooled Ter119<sup>−</sup>/CD31<sup>−</sup>/CD45<sup>−</sup>/CD24<sup>+</sup>/EpCAM<sup>+</sup> cells from three livers = 3,600 vs. 1,200 cells, respectively; Fig. 4G). Following macrophage injection, we found an increase in cells with HPC-defining characteristics *i.e.*, colony formation from clonal density growth *in vitro* with the resultant cells of both a hepatocellular and biliary morphology (Fig. 4H) that could be maintained in the long term and passaged. A total of six viable clonal colonies were obtained after 2 mo from 3,600 sorted Ter119<sup>−</sup>/CD31<sup>−</sup>/CD45<sup>−</sup>/CD24<sup>+</sup>/EpCAM<sup>+</sup> cells in the macrophage



**Fig. 3.** Macrophage transfer recapitulates whole BMC transfer. Twenty-one days following infusion of  $10^7$  syngenic macrophages expansion of panCK<sup>+</sup> DRs is observed, reproducing both morphologically (A) and quantitatively (B) the expansion seen with BMC (each group  $n = 5$ ). Expansion of A6<sup>+</sup> and MIC1-1C3<sup>+</sup> DRs is also observed (C and Inset, respectively), together with active proliferation of Ki67<sup>+</sup>/panCK<sup>+</sup> ductular cells (D). (E) Raised serum albumin is observed 21 d following macrophage transfer. (F) Macrophage depletion was performed by delivering  $10^7$  BMCs from CD11b-DTR mice to WT littermates. DT was given on 0, 3, and 6 d post-BMC transfer to deplete donor-derived macrophages. Tissue was analyzed at 21 d ( $n \geq 5$  each group). Control mice received PBS injection instead of DT. (G) Following transfer of  $10^7$  BMCs from CD11b-DTR donors. Data are presented mean  $\pm$  SEM,  $P$  values denote two (E) and one (B and G) tailed Student  $t$  test.







(16). We now show that exogenous TWEAK administration results in DR expansion in vivo and in addition confirm TWEAK expression by hepatic macrophages and Fn14 expression by panCK<sup>+</sup> DRs, which have been shown to mediate ductular proliferation via NF $\kappa$ B activation (16, 17). Herein we demonstrate a direct link between macrophage TWEAK production and paracrine signaling to recipient DRs, resulting in their expansion using complimentary TWEAK/Fn14 KO models. Similar inhibition of ductular expansion in both KO scenarios is in keeping with the monogamous relation between this signal–receptor pair (31). Ductular expansion has previously been shown to be possible in the absence of Fn14 expression (17). Regarding the localization of macrophages to the biliary cells, previous data has described the recruitment of C-X-C chemokine receptor 4 (CXCR4<sup>+</sup>) cells to the biliary tracts via the chemokine Stromal Cell Derived Factor (SDF)-1 (32, 33). Macrophages strongly express CXCR4, and this may be a mechanism responsible for cell trafficking in this model. Interestingly the injected macrophages recruited endogenous macrophages to the liver, which may act to amplify any macrophage-derived signal.

Following tissue injury, macrophages can modulate progenitor cell behavior, suggesting a role for these cells in linking injury to regeneration (4, 19, 20, 34). Our observations both support and extend this, crucially showing that macrophages are able to modulate DRs even in the absence of injury. We also describe TWEAK as a central molecular target for future trophic/small

molecule therapy. Moreover a therapeutic approach to tissue regeneration based on macrophage therapy offers several advantages: the ready availability of donor cells from an autologous source; the feasibility of the approach; and a defined mechanism of action involving paracrine signaling between donor and host liver cells, without the disadvantages associated with exogenously administered stem cells or their derivatives.

## Materials and Methods

Animals were housed in a specific pathogen free environment and kept under standard conditions with a 12 hour day/night cycle and access to food and water ad libitum. All animal experiments had local ethical approval and were conducted under UK Home Office Legislation. Recipients received  $1 \times 10^7$  BMCs via tail vein injection. For details of the animal models used, macrophage differentiation protocol, harvest of transferred cells, immunohistochemistry, fluorescence in situ hybridization, real time PCR, statistical analysis, microscopy and cell counting methodology please see [SI Materials and Methods](#).

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